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Originals

Phylogeography of *Stachyurus praecox* (Stachyuraceae) in the Japanese Archipelago Based on Chloroplast DNA Haplotypes

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The phylogeographical structure of chloroplast DNA haplotypes within *Stachyurus praecox* Siebold & Zucc. (Stachyuraceae) was investigated throughout the species range by comparing the sequences of two intergenic regions, the *trnT*(UGU)-*trnL*(UAA)5'exon and the *trnL*(UAA)5'exon-*trnF*(GAA). Eleven cpDNA haplotypes were found within *S. praecox*. These haplotypes appeared to be phylogeographically structured. Whereas most haplotypes were concentrated on the Pacific Ocean side of Japan and each haplotype showed a localized distribution, one haplotype ranged widely in the northern part of the archipelago and the Sea of Japan side. Four haplotypes in the eastern part of the Pacific Ocean side formed a lineage group. By integrating the geographical distribution and the genealogical relationship of haplotypes, the species range can be divided into at least three major geographical regions; the western part of the Pacific Ocean side, the eastern part of the Pacific Ocean side, and the region from the northern part of the archipelago to the Sea of Japan side. This apparent phylogeographical structure was interpreted to be the result of distribution changes effected by climatic oscillations from the late glacial period to the present.

Key words: chloroplast DNA, intraspecific variation, phylogeography, *Stachyurus praecox*, the Japanese archipelago.

Intraspecific phylogeography provides new insights into species history based on the phylogeographical structure, which can be recognized by identification of the geographical distribution and the genealogical relationship of mitochondrial or chloroplast haplotypes through a species' range (Avise et al. 1987, Avise 2000). So far most of phylogeographical studies have been conducted on North American species (e.g., Sewell et al. 1996, Soltis et al. 1997), and widespread European tree species (e.g., Ferris et al. 1993, 1995, Demesure et al. 1996, Petit et al. 1993, 1997, Dumolin-Lapègue et al. 1997, King and Ferris 1998, Sinclair et al. 1999). These studies have suggested that the phylogeographical structure of haplotypes has been strongly influenced by the climatic oscillations of the last glacial period (Hewitt 1996, 2000). In spite of the recent explosion in phylogeographic research, only a few studies have been reported for Japanese plants; Japanese beech (*Fagus crenata*; Tomaru et al. 1998, Fujii et al.

2002), Japanese firs (*Abies* spp.; Tsumura and Suyama 1998), and Japanese alpine plants (Fujii et al. 1997, 1999).

The Japanese archipelago is located on the eastern edge of East Asia and consists of main islands (Hokkaido, Honshu, Shikoku, and Kyushu), which extend from northeast to southwest over a distance of about 3,000 km (Fig. 1). The archipelago includes biomes ranging from subtropical to cool-temperate. As part of a long-term study, we focused on elucidating the phylogeographical structure of those common, widespread species that have continuous distributions from evergreen forest to broadleaf deciduous forest in temperate zones of the Japanese archipelago. As the first representative, we selected an endemic deciduous shrub, *Stachyurus praecox* Siebold & Zucc. (Stachyuraceae). The species is widely distributed from southwest Hokkaido, throughout Honshu, Shikoku, and Kyushu, to Tokunoshima Island in the northern part of the Nansei Islands, (Horikawa 1972) (Fig. 1). Within the species, three varieties, var. *matsuzakii* (Nakai) Makino; var. *lancifolius* (Koidz.) H.Hara and var. *leucotrichus* Hayashi, have been recognized (Ohba 1999). However it is often difficult to identify infraspecific taxa because of the observed clinal morphological variation.

In the course of a molecular study to produce our previous report, which aimed to discuss the origin of the endemic evergreen species in the Ogasawara Islands (viz., *Stachyurus macrocarpus* Koidz.), we noticed that two intergenic regions of cpDNA, the *trnT* (UGU)-*trnL* (UAA) 5'exon and the *trnL* (UAA) 5'exon-*trnF* (GAA), showed considerable intraspecific variation within *S. praecox* (Ohi and Murata 2000). However, sampling localities were not sufficient to grasp the phylogeographical structure of haplotypes. In addition, in order to discuss the phylogeographical history of the species, it is necessary to analyze related species from Taiwan, which is considered geographically and floristically close to the Japanese archipelago and thus is important from the point of view of phytogeography. In the present study, we investigate the phylogeographical structure of cpDNA haplotypes throughout the species range by increasing sampling from widespread Japanese localities, and adding the related species *S. himalaicus* Hook.f. & Thomson and *S. sigeyosii* Masam. from Taiwan.

Materials and Methods

As our aim was to grasp the outline of the phylogeographical structure within *S. praecox*, we decided to sample one or two plants from many localities rather than many from several populations, irrespective of morphology. We sampled a total of 105 plants of *S. praecox* from 101 locations, including an additional 56 samples beyond the 49 samples used in the previous analysis (Table 1). The sample locations sufficiently covered the entire natural range of the species. In addition to *S. macrocarpus* and *S. chinensis* Franch., which were also used in the previous analysis, *S. himalaicus* Hook.f. & Thomson and *S. sigeyosii* Masam. from Taiwan, were used as outgroups (Table 1). Voucher specimens were deposited in the Herbarium of the University of Tokyo (TI).

Leaves of *Stachyurus* contain a large amount of sticky polysaccharide. Prior to genomic DNA extraction, therefore, dried leaf powder was suspended in HEPES buffer (pH 8.0) and centrifuged at 10,000 rpm of 20 °C for 5 min to completely remove the sticky polysaccharides, following the method of Setoguchi and Ohba (1995). Genomic DNA was extracted from the resulting pellets using the CTAB method of Hasebe and Iwatsuki (1990).

Two intergenic regions of cpDNA, the *trnT* (UGU)-*trnL* (UAA) 5'exon and the *trnL* (UAA) 5'exon-*trnF* (GAA) were sequenced for additional samples. The two regions were amplified using the polymerase chain reac-

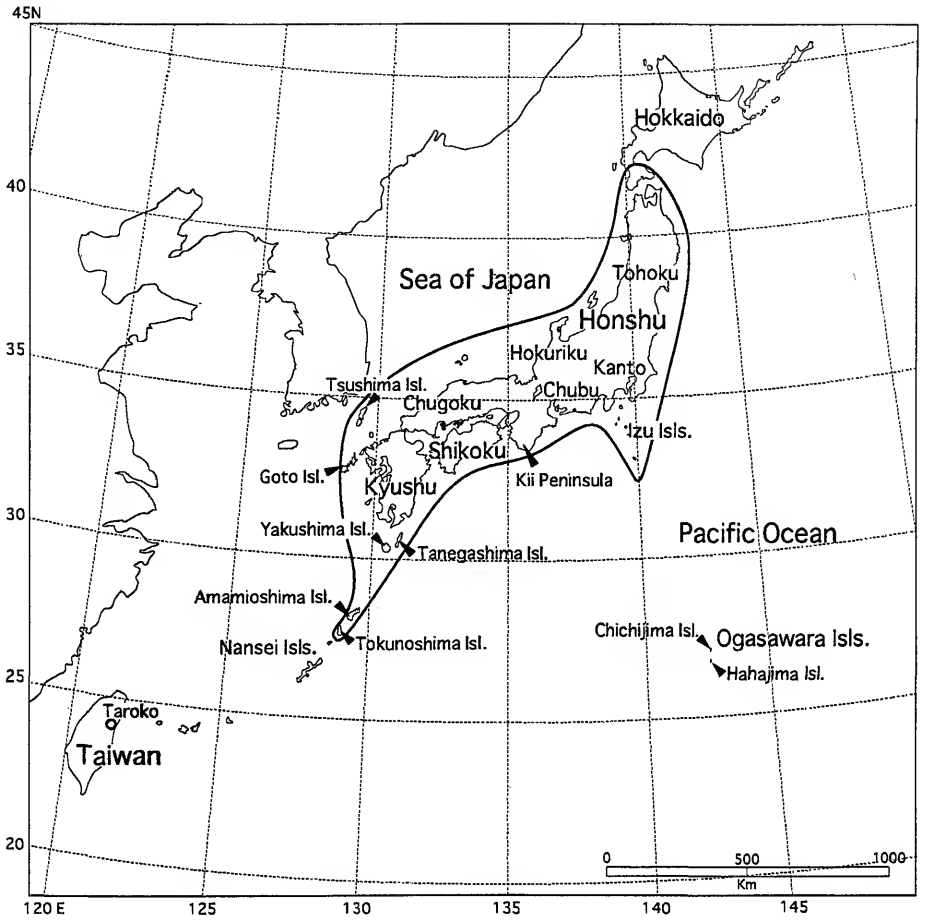

Fig. 1. Present distribution of *Stachyurus praecox*, and names of districts and regions in the Japanese archipelago and Taiwan. *Stachyurus macrocarpus* and *S. sigeyosii* are endemic to the Ogasawara Islands, and to Taroko in Taiwan, respectively.

tion (PCR). The PCR reaction mixture consisted of 1.0 unit of ExTaq polymerase (TaKaRa), 10 × PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM of each dNTP, 5 pmol of each primer, 1–30 ng of genomic DNA, in a total volume of 30 µl. PCR cycling conditions were 96 °C (1 min); then 40 cycles of 96 °C (1 min), 55 °C (2 min), 72 °C (2 min); and finally 72 °C (10 min). The universal primer sets of Taberlet et al. (1991); a (5'-CATTA CAAATGCGATGCTCT-3') and b (5'-TCT ACCGATTTCGCCATATC-3'), c (5'-CGA AATCGGTAGACGCTACG-3') and f (5'-ATTTGAACTGGTGACACGAG-3') were used. PCR products were purified by electrophoresis in 1.0 % TAE agarose gel stained with ethidium bromide, and GeneClean II DNA purification Kit (BIO 101). Purified fragments were cycle-sequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems) following the supplier's instructions. DNA sequences were obtained using an ABI PRISM 377 DNA Sequencer (Perkin-Elmer Applied Biosystems).

Table 1. Sample information and cpDNA haplotypes in *Stachyurus* species

Species, sample number and locality		Latitude	Longitude	Altitude (m)	Voucher in TI	Haplo-type
Stachyurus praecox Siebold & Zucc.						
1	Hokkaido Pref., Hakodate City, Momijiyama Town	41° 49′ N	140° 53′ E	200	SHOK	F
2	Aomori Pref., Ajigasawa Town, Nagahira	40° 42′ N	140° 16′ E	350	SIWK*	F
3	Aomori Pref., Takko Town, Shimizugashira,	40° 21′ N	141° 07′ E	300	STKK	F
4	Iwate Pref., Esashi City, Kawachi	39° 07′ N	141° 02′ E	300	SESS	F
5	Iwate Pref., Kuzumaki Town, Urashinai	39° 58′ N	141° 20′ E	500	SKZM*	F
6	Miyagi Pref., Naruko Town, Matsusawa	38° 45′ N	140° 43′ E	300	SNRK*	F
7	Akita Pref., Chokai Town, Momoyake	39° 06′ N	140° 10′ E	550	SCKI	F
8	Akita Pref., Rokugo Town, Mt. Kuromori	39° 23′ N	140° 38′ E	450	SKRM	F
9	Akita Pref., Moriyoshi Town, Mt. Moriyoshi	40° 02′ N	140° 10′ E	400	SMRY*	F
10	Yamagata Pref., Yamagata City., Kamisakurada	38° 13′ N	140° 20′ E	350	SZAO	F
11	Fukushima Pref., Yanagawa Town, Kabuto	37° 53′ N	140° 40′ E	100	SABK*	H
12	Fukushima Pref., Iwaki City., Shimonagai	37° 08′ N	140° 46′ E	250	SFKS	J
13	Fukushima Pref., Kitakata City, Negoya	37° 44′ N	139° 56′ E	600	SKTK	F
14	Fukushima Pref., Tajima Town, Mt. Takakura	37° 09′ N	139° 42′ E	900	STJM	F
15	Ibaraki Pref., Yasato Town, Mt. Wagakuni	36° 19′ N	140° 12′ E	350	SYST*	H
16	Tochigi Pref., Ashio Town, Nakasai	36° 39′ N	139° 25′ E	800	SASO*	K
17	Saitama Pref., Ogano Town, Mt. Futago	36° 04′ N	138° 53′ E	800	SFTG	J
18	Chiba Pref., Maruyama Town, Miyashita	35° 03′ N	139° 55′ E	100	SBSO	I
19	Chiba Pref., Ichihara City, Arai	35° 20′ N	140° 11′ E	150	SICH	J
20	Chiba Pref., Tomiura Town, Tatara	35° 02′ N	139° 50′ E	20	SOFU*	J
21	Chiba Pref., Nado, Taiei Town	35° 50′ N	140° 25′ E	50	STEI*	I
22	Tokyo Pref., Hachijojima Island of the Izu Isls.	36° 06′ N	139° 46′ E	300	SHJO*	I
23	Tokyo Pref., Miyake Island of the Izu Isls.	34° 04′ N	139° 29′ E	20	SMIY*	I
24	Tokyo Pref., Oume City, Kurosawa	35° 48′ N	139° 15′ E	400	SOUM*	H
25	Kanagawa Pref., Kamakura City, Jyunisyo	35° 19′ N	139° 35′ E	150	SKAM*	I
26	Kanagawa Pref., Kamakura City, Koshigoe	35° 18′ N	139° 29′ E	50	SSCR	J
27	Kanagawa Pref., Manatsuru Town	35° 08′ N	139° 08′ E	20	SMND*	I
28	Kanagawa Pref., Yamakita Town	35° 24′ N	139° 04′ E	600	STZW	J
29	Niigata Pref., Nagaoka City, Mt. Kazetani	37° 23′ N	138° 55′ E	300	SNGT*	F
30	Niigata Pref., Sanpoku Town	38° 28′ N	139° 33′ E	180	SSPK*	F
31	Niigata Pref., Itoigawa City, Hiraiwa	36° 53′ N	137° 52′ E	350	SKOT	F
32	Toyama Pref.,Yatsuo Town, Mt. Shirakimine	36° 25′ N	137° 07′ E	1,100	SSRK 1*	G
33	Toyama Pref.,Yatsuo Town, Mt. Shirakimine	36° 25′ N	137° 07′ E	950	SSRK 2	G
34	Toyama Pref., Himi City	36° 51′ N	136° 57′ E	100	STYM	F
35	Yamanashi Pref., Mitama Town, Nakayama	35° 32′ N	138° 32′ E	350	SMTM*	J
36	Nagano Pref., Chino City, Ankokuji	35° 59′ N	138° 10′ E	950	SCNO	J
37	Nagano Pref., Sanada Town, Sugadaira	36° 32′ N	138° 17′ E	1,100	SSGD	F
38	Nagano Pref., Azumi Vill., Sawando	36° 10′ N	137° 38′ E	1,000	SSWD*	J
39	Gifu Pref., Gero Town, Natsuyake	35° 44′ N	137° 16′ E	500	SGRO	F
40	Gifu Pref., Shirakawa Vill.	36° 13′ N	136° 53′ E	1,000	SHAK*	F
41	Shizuoka Pref., Ochii, Minamiizu Town	34° 41′ N	138° 49′ E	200	SIZU*	J
42	Shizuoka Pref., Daito Town, Mt. Ogasa	34° 44′ N	137° 38′ E	150	SOGS*	J
43	Shizuoka Pref., Honkawane Town, Tomisawa	35° 06′ N	137° 31′ E	400	SOOI	J
44	Shizuoka Pref., Nakaizu Town, Mt. Togasa	34° 42′ N	139° 04′ E	900	STGS	J
45	Aichi Pref., Tsugu Vill., Mt. Chausu	35° 13′ N	137° 39′ E	1,300	SCUS	K
46	Aichi Pref., Horai Town, Mt. Horaiji	34° 57′ N	137° 34′ E	130	SHRJ*	J
47	Mie Pref., Komono Town, Yunoyama	35° 59′ N	135° 50′ E	250	SKMN	F
48	Mie Pref., Miyama Town, Shiroura	34° 07′ N	136° 17′ E	10	SUMY*	I
49	Shiga Pref., Kutsuki Vill., Ueno	35° 22′ N	135° 53′ E	200	SSGA	F
50	Shiga Pref., Kinomoto Town, Yakusa	35° 34′ N	136° 20′ E	700	SYKS	F
51	Kyoto Pref., Miyatsu City, Hata	35° 36′ N	135° 12′ E	150	SKYO*	F
52	Kyoto Pref., Wazuka Town, Yubune	34° 49′ N	135° 56′ E	300	SWDK*	J
53	Hyogo Pref., Aioi City, Mt. Minou	34° 54′ N	134° 27′ E	250	SAIO	F
54	Hyogo Pref., Okutoji, Asago Town	34° 13′ N	134° 14′ E	600	SASG	F
55	Hyogo Pref., Sumoto City, Awajishima Island	34° 18′ N	134° 55′ E	200	SAWJ	I
56	Hyogo Pref., Kobe City, Mt. Rokko	34° 47′ N	135° 16′ E	450	SRKO	F
57	Nara Pref., Nosegawa Vill., Mt. Kojin	34° 09′ N	135° 38′ E	1,000	SKJN*	F
58	Wakayama Pref., Hidaka Town, Oura	33° 55′ N	135° 04′ E	10	SHDK*	I
59	Wakayama Pref., Hongu Town, Minasegawa	33° 48′ N	135° 46′ E	200	SHNG	F
60	Wakayama Pref., Kushimoto Town, Wabuka	33° 29′ N	135° 40′ E	50	SKSM*	I
61	Shimane Pref., Ohta City, Sojiki Town	35° 03′ N	132° 27′ E	350	SYAK*	A
62	Okayama Pref., Chuka Vill., Mt. Yamanori	35° 14′ N	133° 49′ E	600	SOOT	F
63	Okayama Pref., Yoshii Town, Yamamura	34° 39′ N	133° 24′ E	150	SYNY*	F
64	Hiroshima Pref., Ohno Town, Mt. Kyogoya	34° 19′ N	132° 16′ E	200	SYSI	F

Table 1. (continued)

Species, sample number and locality	Latitude	Longitude	Altitude (m)	Voucher in TI	Haplo-type
65 Hiroshima Pref., Takamiya Town, Funaki	34° 47′ N	132° 44′ E	200	SHRS	F
66 Yamaguchi Pref., Tokuji Town, Yugi	34° 20′ N	131° 43′ E	300	STMY*	F
67 Yamaguchi Pref., Yuya Town, Jarigatao	34° 18′ N	131° 04′ E	400	STKD*	F
68 Tokushima Pref., Shishikui Town, Shishikuiura	33° 33′ N	134° 18′ E	20	SYMG	F
69 Tokushima Pref., Kisawa Vill., Tosu	33° 54′ N	134° 15′ E	800	SSSK	I
70 Kagawa Pref., Kotonami Town, Kawahigashi	34° 07′ N	133° 58′ E	300	STOS*	E
71 Ehime Pref., Ikata Town	33° 37′ N	132° 20′ E	100	SKTN	E
72 Ehime Pref., Hiromi Town, Ohjiku	33° 20′ N	132° 41′ E	200	SEHI*	F
73 Ehime Pref., Iyomishima City, Iwanabe	33° 55′ N	133° 32′ E	380	SHRM	C
74 Ehime Pref., Hojo City, Kugawa	33° 54′ N	132° 52′ E	400	SIYO	F
75 Kochi Pref., Kagami Town, Betchaku	33° 36′ N	133° 48′ E	200	SKKW	C
76 Kochi Pref., Muroto City, Murotsu	33° 20′ N	134° 10′ E	100	SKGM	E
77 Kochi Pref., Agawa Vill., Mt. Myojin	33° 34′ N	133° 04′ E	800	SMRT*	I
78 Kochi Pref., Nakatosa Town, Nagasawadani	33° 19′ N	133° 13′ E	200	SMYO*	C
79 Kochi Pref., Sukumo City, Sakanoshita	32° 55′ N	132° 43′ E	10	SNTS	F
80 Fukuoka Pref., Kitakyushu City, Masubuchi	33° 44′ N	130° 50′ E	300	SSKM*	C
81 Fukuoka Pref., Nakagawa Town, Minamihata	33° 26′ N	130° 14′ E	300	SKKS*	F
82 Nagasaki Pref., Chijiwa Town, Tashirohara	32° 47′ N	130° 14′ E	650	SNKG	D
83 Nagasaki Pref., Kishiku Town, Fukuejima Island	32° 43′ N	128° 44′ E	30	SCDW	F
84 Nagasaki Pref., Tamanoura Town, Fukuejima Island	32° 37′ N	128° 41′ E	40	SFKE 1	B
85 Nagasaki Pref., Hirado City, Hiradojima Island	33° 20′ N	129° 28′ E	80	SFKE 2	B
86 Nagasaki Pref., Hirado City, Hiradojima Island	33° 11′ N	129° 22′ E	50	SHRD 1*	B
87 Nagasaki Pref., Ohmura City, Mt. Tara	32° 59′ N	130° 12′ E	800	SHRD 2	B
88 Nagasaki Pref., Izuhara Town, Tsushima Island	34° 09′ N	129° 14′ E	100	STAR*	D
89 Kumamoto Pref., Hitoyoshi City, Kushichi	32° 07′ N	130° 40′ E	500	STSM	D
90 Kumamoto Pref., Ushibuka City, Futaura Town	32° 16′ N	130° 01′ E	20	SKSC*	D
91 Kumamoto Pref., Tomochi Town, Hayatachibana	32° 34′ N	130° 52′ E	900	SUSB*	A
92 Oita Pref., Bungotakada City, Ichihata	33° 33′ N	131° 34′ E	500	STMC	D
93 Oita Pref., Ume Town, Mt. Kashiwa	32° 54′ N	131° 33′ E	500	SBNG	F
94 Oita Pref., Nakatsue Vill., Syukugamineo	33° 04′ N	130° 49′ E	700	SKSW	D
95 Miyazaki Pref., Nobeoka City, Yasui	32° 36′ N	131° 45′ E	50	SSKG*	D
96 Miyazaki Pref., Tsuno Town, Mt. Osuzu	32° 17′ N	131° 00′ E	600	SNBO*	C
97 Miyazaki Pref., Tano Town, Ohtonogoe	31° 47′ N	131° 18′ E	400	SOSZ	D
98 Kagoshima Pref., Amamioshima Island, Mt. Kochi	28° 11′ N	129° 21′ E	400	STNO	D
99 Kagoshima Pref.,Yamato Vill., Amamioshima Island	28° 19′ N	129° 17′ E	50	SAMA 1*	B
100 Kagoshima Pref., Ei Town, Yukimaru	31° 16′ N	130° 33′ E	200	SAMA 2	B
101 Kagoshima Pref., Kirishima Town, Yunotani	31° 53′ N	130° 51′ E	600	SIBS	E
102 Kagoshima Pref., Sendai City, Yorita Town	31° 47′ N	130° 11′ E	20	SKRS	D
103 Kagoshima Pref., Minamitane Town, Tanegashima Island	30° 27′ N	130° 52′ E	50	SSND*	A
104 Kagoshima Pref., Umikura, Uchinoura Town	31° 19′ N	131° 08′ E	800	STAN*	E
105 Kagoshima Pref., Kamiyaku Town, Yakushima Island	30° 23′ N	130° 37′ E	300	SUCU*	E
Stachyurus macrocarpus Koidz.					
106 Tokyo Pref., Chichijima Island of Ogasawara Isls.	27° 04′ N	142° 12′ E	300	SNAG 1*	L
107 Tokyo Pref., Chichijima Island of Ogasawara Isls.	27° 04′ N	142° 12′ E	200	SNAG 2*	L
108 Tokyo Pref., Hahajima Island of Ogasawara Isls.	26° 39′ N	142° 09′ E	440	SHAZ*	M
Stachyurus sigeyosii Masam.					
109 Taiwan, Hualien Hsien,Taroko, Meiyuan	24° 12′ N	121° 28′ E	900	STW 58	N
110 Taiwan, Hualien Hsien,Taroko, Liushui	24° 10′ N	121° 30′ E	450	STW 68	N
111 Taiwan, Hualien Hsien,Taroko, Chiuchu Tung	24° 31′ N	121° 31′ E	400	STW 70	N
Stachyurus himalaicus Hook.f. & Thomson					
112 Taiwan, Kaohsiung Hsien, Tengchi, Shih Shan	23° 03′ N	120° 45′ E	1,450	STW 21	X 1
113 Taiwan, Kaohsiung Hsien, Tengchi, Chuyun Shan	23° 03′ N	120° 46′ E	1,000	STW 27	X 2
114 Taiwan, Nantou Hsien, Mei Feng	24° 05′ N	121° 10′ E	2,200	STW 33	X 3
115 Taiwan, Hualien Hsien,Taroko, Meiyuan	24° 12′ N	121° 28′ E	900	STW 62	X 1
Stachyurus chinensis Franch.					
116 cult. in Nikko Botanical Garden of The Univ. of Tokyo	–	–	–	A 2376*	CHI

Asterisks (*) indicate plants used in the previous report (Ohi and Murata 2000).

Haplotypes were identified based on the combined sequences of *trnT-trnL* and *trnL-trnF*. Multiple sequence alignment and gap coding were performed manually. The following criteria alignment and interpretations of mutation were used (modified from Golenberg et al. 1993, Kelchner 2000). (1) If a gap was the result of an insertion or a deletion of a repeat unit suggesting alternative positions, the gap was placed so as to minimize nucleotide mismatches. (2) A gap of equal length shared by two or more sequences or a gap arising from the duplication of a neighboring sequence was considered to have arisen by a single mutational event. (3) When two or more gaps were not identical but overlapped, the overlapping portions of gaps were considered shared events only when the region could be partitioned into informative insertion or duplication regions on at least one side. (4) The informative gaps were coded as binary figures of "0 or 1" and scored as unordered characters. (5) Gaps as part of polynucleotide tracts (polyA or polyT) were excluded as uninformative characters.

The genealogical relationships of haplotypes was inferred by parsimony analysis using PAUP* version 4.0 (Swofford 2001), using the heuristic search option, random addition sequence with 100 replicates, TBR branch swapping, and MULTREES options on. Bootstrap analysis of 1,000 replicates was conducted to assess the internal support. Then, in order to efficiently show the relationships of haplotypes, the strict consensus tree of the most parsimonious trees was transformed into a minimum spanning tree. Because intraspecific gene genealogies are often multifurcated, the intraspecific genealogy cannot always be represented by a bifurcating tree (Posada and Crandall 2001). Thus, the minimum spanning tree is an alternative to Wagner parsimony tree that better conveys the connections between haplotypes within species (Excoffier et al. 1992, Schaal and Olsen 2000).

Results

Chloroplast DNA variation

In addition to the previous data of Ohi and Murata (2000), *trnT-trnL* and *trnL-trnF* sequences were newly determined for 56 individuals of *S. praecox*, four of *S. himalaicus* and three of *S. sigeyosii*. Sequences were deposited in the DDBJ/EMBL/GenBank databases (accession numbers AB066300 to AB066335). Within *S. praecox*, the length of *trnT-trnL* ranged from 1,188 to 1,222 bp (GC content 22.94–23.24 %) and the length of *trnL-trnF* ranged from 959 to 969 bp (GC content 35.19–35.45 %). The former region possessed higher levels of genetic variation (four substitutions and five gaps) than the latter (one substitution and one gap) (Tables 2, 3). In these combined sequences (2,147–2,184 bp), variable characters included five substitutions and six gaps. All gaps were tandem repeats of neighboring random sequences resulting from slippage, their sizes ranging from 8 to 26 bp (Table 3). By adding new samples, no new haplotypes were found in the present analysis, and consequently, eleven haplotypes (A through K) were identified (Table 1 and 3). As regards outgroup species, two haplotypes (L and M) were found within *S. macrocarpus,* and three haplotypes (X1, X2 and X3) within *S. himalaicus*. *Stachyurus sigeyosii* (halplotype N) and *S. chinensis* were also found to have a specific haplotypes (Tables 1, 3).

Geographical distribution of cpDNA haplotypes

Each of the haplotypes, when plotted on a map, were localized in a particular area (Fig. 2). Haplotype A (three locations; 2.85 %) appeared in the coastal area of southwest Kyushu and on Yakushima Island. Haplotype B (six locations; 5.71 %) appeared on the islands of northwest Kyushu and on Amamioshima Island. Haplotype C (five lo-

Table 2. Sequence length, GC content, and polymorphic characters of two intergenic region in *Stachyurus* species

Intergenic region of cpDNA	within *S. praecox*				within all accessions			
	Length (bp)	GC content (%)	Substi-tutions	Gaps*	Length (bp)	GC content (%)	Substi-tutions	Gaps*
trnT(UGU)–*trnL*(UAA)5'exon	1,188–1,222	22.94–23.24	4	5	1,149–1,226	22.54–23.24	12	21
trnL(UAA)5'exon-*trnF*(GAA)	959– 969	35.19–35.45	1	1	959– 985	34.51–35.45	5	3
totall	2,147–2,184	28.36–28.64	5	6	2,112–2,188	28.20–28.64	17	24

*Gaps as part of polynucleotide tracts were excluded as uninformative characters.

Table 3. CpDNA haplotypes and their mutations in *Stachyurus* species

CpDNA haplotype*	Substitutions**						Gaps and their size (bp)**									No. of plants	Frequency (%)
	S1	S2	S3	S4	S5	S6	G1	G2	G3	G4	G5	G6	G7	G8	G9		
A	G	T	A	G	G	C	–	–	–	–	–	–	–	–	–	3	2.86
B	G	T	A	G	G	C	–	–	–	–	–	–	–	26	–	6	5.71
C	G	T	A	G	G	C	17	–	–	–	–	–	–	–	–	5	4.76
D	G	T	A	A	G	A	–	–	–	–	–	–	–	–	–	10	9.52
E	G	G	A	G	G	C	–	16	–	–	–	–	–	–	–	6	5.71
F	G	T	A	G	G	C	–	–	–	–	–	–	8	–	–	41	39.05
G	G	T	A	G	G	C	–	–	–	–	20	–	8	–	–	2	1.90
H	G	T	C	G	G	C	–	–	–	–	–	–	–	–	–	3	2.86
I	G	T	C	G	G	C	–	–	–	–	–	–	–	–	9	12	11.43
J	T	T	C	G	G	C	–	–	–	–	–	–	–	–	–	15	14.29
K	T	T	C	G	G	C	–	–	–	–	–	–	8	–	–	2	1.90
L	G	T	A	G	G	C	–	–	14	–	–	–	–	–	–	2	–
M	G	T	A	G	G	C	–	–	14	14	–	–	–	–	–	1	–
N	G	T	A	G	A	C	–	–	–	–	–	17	8	–	–	3	–

*Haplotypes of *S. chinensis* and *S. himalaicus* were excluded.

**Substitutions (S) and gaps (G) were numbered in order from 5' end of *trnT-trnL* to 3' end of *trnL-trnF*, respectively. Of them, S6 and G9 were found in *trnL-trnF*.

cations; 4.76 %) mostly appeared in the western part of Shikoku. Haplotype D (10 locations; 9.52 %) appeared in the mountainous region of Kyushu and on Tsushima Island. Haplotype E (six locations; 5.71 %) showed a disjunctive distribution on southern Kyushu and on Tanegashima Island, and also in eastern Shikoku. Haplotype F was found most frequently, in 41 locations (39.05 %), extending widely from southwestern Hokkaido, through Tohoku District to the Sea of Japan side in Chugoku District and into the central Kii Peninsula of Honshu. The haplotype also appeared in parts of Kyushu and Shikoku. Haplotype G was found in two plants (1.90 %) from only one location, Mt Shirakimine in the Hokuriku District. Haplotype H (three locations; 2.85 %) appeared from Tokyo to southeastern Tohoku along the Pacific Ocean side. Haplotype I (12 locations; 11.43 %) showed a disjunct distribution; in the coastal area of the Kii Peninsula and southeastern Shikoku, and also in Kanto District and the Izu Islands.

Haplotype J (15 locations; 14.29 %) appeared from Kanto District to the southern Chubu region. Haplotype K appeared in two separate locations (1.90 %). In summary, most of the haplotypes (A–E and H–K) were concentrated on the Pacific Ocean side, from southeastern Tohoku throughout Honshu to Shikoku and Kyushu, and the Izu Islands and the northern part of the Nansei Islands, whereas the single major haplotype F was widely distributed through the northern part of the archipelago and the Sea of Japan side, with a rare haplotype G occurring in the middle.

Genealogical relationships among cpDNA haplotypes

Among all accessions including the outgroups, the total lengths of combined sequences for these regions ranged from 2,112 to 2,188 bp, and the aligned sequence length was 2,443 bp. A total of 41 characters, 17

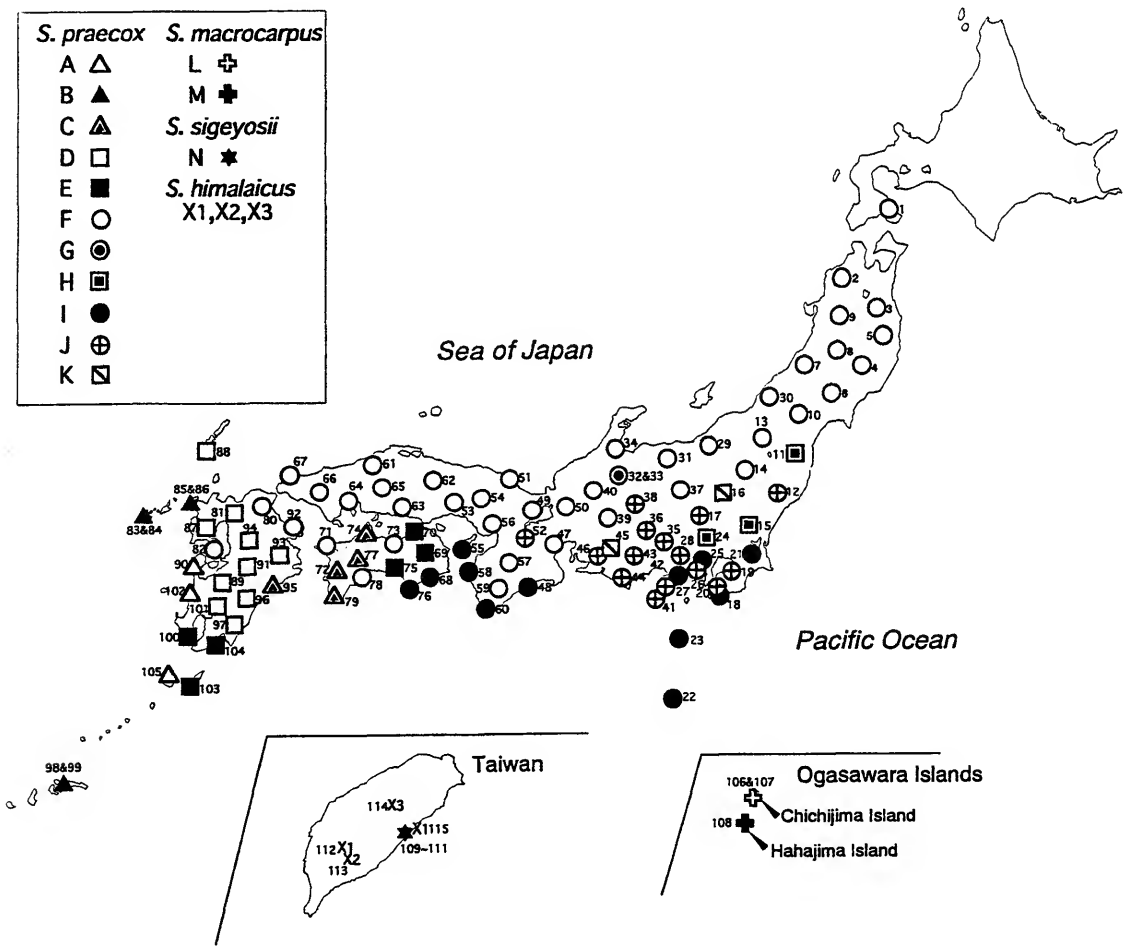

Fig. 2. Geographical distribution of cpDNA haplotypes based on the sequences of two intergenic regions, *trnT*(UGU)–*trnL*(UAA)5'exon and *trnL*(UAA)5'exon–*trnF*(GAA). Haplotypes A through K are of *Stachyurus praecox*; haplotype L and M are of *S. macrocarpus*; haplotype N is of *S. sigeyosii*; and haplotype X1, X2 and X3 are of *S. himalaicus*. Numbers with symbols of haplotypes indicate the number of samples, as designated in Table 1.

substitutions and 24 gaps, were variable (Table 2), and of them 21 characters were parsimony informative. Three most parsimonious trees of 43 steps with a consistency index of 0.953 and a rescaled consistency index of 0.923 were obtained. In the strict consensus tree, *S. praecox*, *S. macrocarpus*, and *S. sigeyosii* formed a clade (BP 85 %). In the minimum spanning tree, haplotype A was connected to the clade of *S. himalaicus* and *S. chinensis*, and other haplotypes were connected to it by a few mutational steps (Fig. 3). There were two subgroups but the internal supports of each branch was weak; one consisting of haplotypes H through K (BP 53 %) and another of haplotypes L and M of *S. macrocarpus* (BP 63 %).

Discussion

Stachyurus praecox displayed eleven

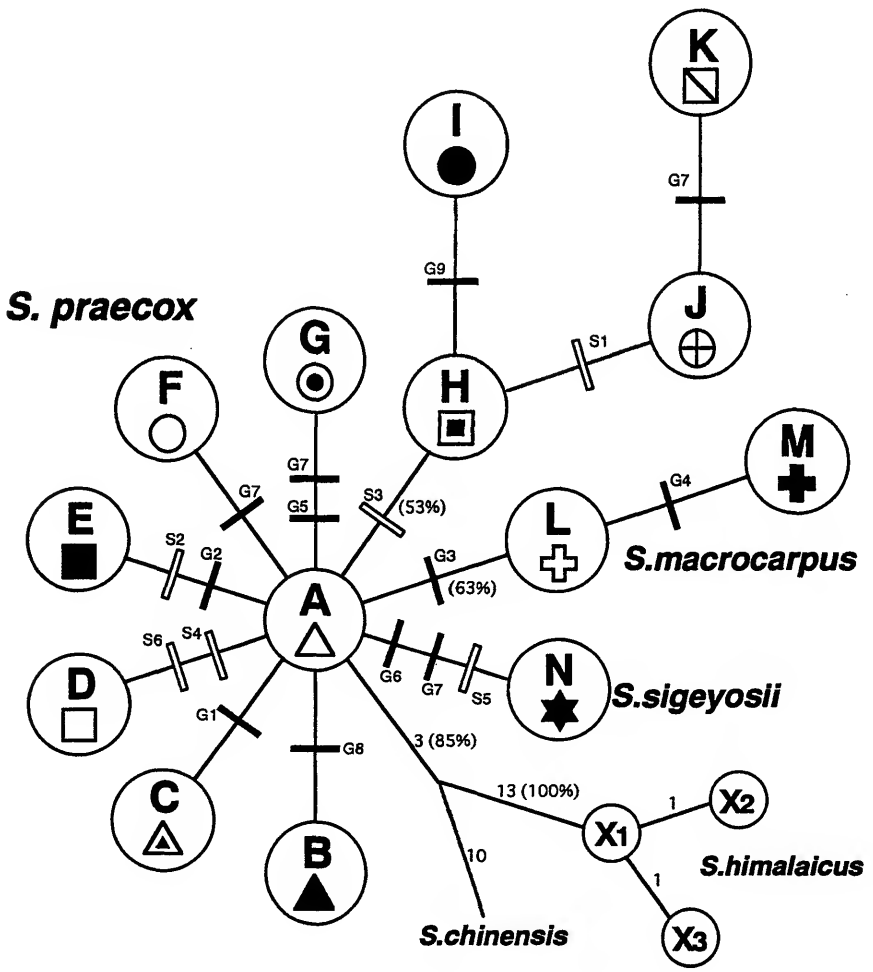

Fig. 3. Minimum spanning tree of cpDNA haplotypes, which was transformed from the strict consensus tree of three most parsimonious trees in *Stachyurus* species. Haplotypes are represented by circles. On each ingroup branch, bootstrap values and mutations are shown; open-bars are substitutions (S1–S6) and closed-bars are gaps (G1–G9), designated as in Table 3. On outgroup branches, bootstrap values and branch lengths are shown.

cpDNA haplotypes (A through K) according to mutations in the sequences of two intergenic regions, *trnT-trnL* and *trnL-trnF* (Table 3). The addition of new samples, covering the whole range of the species, suggests that the geographical distribution of haplotypes is continuously structured (Fig. 2). A single major haplotype (F) is widely distributed in the northern part of the archipelago and the Sea of Japan side (41 individuals; 39.05 %). The other haplotypes were concentrated on the Pacific Ocean side and each of these haplotypes showed a localized distribution. Of them, four haplotypes, H through K (32 individuals, 30.48 %), localized in the eastern part of the Pacific Ocean side formed a lineage group in the minimum spanning tree, although bootstrap support was weak (Fig. 3). Thus, the phylogeographical structure of *S. praecox* is identified by integration of the geographical distribution and the genealogical relationship of haplotypes. The species range can be divided into at least three major geographical regions. The first region, the northern part of the archipelago and the Sea of Japan side from southwestern Hokkaido through Honshu, was primarily covered by the major haplotype F. The second region is the eastern part of the Pacific Ocean side from southeastern Tohoku, through Kanto and Chubu to the Kii Peninsula and the Izu Islands, where haplotypes H through K (belonging to the same genealogical lineage) are distributed. In the remaining region, the western part of the Pacific Ocean side from Shikoku and Kyushu to the Nansei Islands, five haplotypes A through E (30 individuals, 28.57 %) are distributed.

Palynological evidences in recent glacial period indicate that many species in the temperate zones became extinct throughout most of their range during the glacial period, persisting in refugia, and then recolonizing as it became warmer again (Huntley and Webb III 1988). Hewitt (1996, 2000) suggested that these repeated episodes of contraction and expansion of distribution during the glacial cycles influenced the current genetic structure of species. The historical process of distributional change in a species, especially a postglacial expansion, is commonly estimated based on its integrated paleobotanical and phylogeographical data (Tabelret et al. 1998, Cruzan and Templeton 2000). The current phylogeographical structure observed within *S. praecox* may also have been influenced by climatic oscillations from the late glacial period to the present. However as no pollen records of *S. praecox* have yet been reported, we refer here primarily to the influence of the last glacial period in the Japanese archipelago.

Although the archipelago was not covered by major ice sheets during the last glacial period (Ono 1984), the mean annual temperature was 5–8 °C cooler than at present (Tsukada 1988, Yasuda and Narita 1981). Palynological reconstructions of vegetation indicate that during the last glacial period much of Honshu was covered by boreal conifer forest and a smaller part by boreal deciduous broadleaf forest. It is thought that most temperate plants survived in a limited area along the Pacific Ocean, in southernmost Kyushu, Shikoku and Kanto (Kamei et al. 1981, Tsukada 1988). Considering that the contemporary distribution of *S. praecox* ranges from evergreen warm-temperate forest to broadleaf deciduous cool-temperate forest in temperate zones below the *Fagus* zone (Kanai 1963), it is unlikely that the species could survive during the last glacial period in the major part of Honshu, which was mainly covered by boreal conifer forests (Kamei et al. 1981, Tsukada 1988). It seems reasonable that the refugial areas of *S. praecox* were along the coast of the Pacific Ocean side.

Some phylogeographical studies indicate that contemporary genetic diversity of cpDNA (haplotype richness) in putative

refugial areas is higher than that in nonrefugial areas (Demesure et al. 1996, Dumolin-Lapègue et al. 1997, Soltis et al. 1997, Taberlet et al. 1998). Our results showed that most haplotypes were concentrated in the Pacific Ocean side, i.e. the location of putative refugia areas, whereas a single major haplotype (F) is widely distributed in the northern part of the archipelago and the Sea of Japan side (i.e., nonrefugial areas). This pattern has been interpreted as a consequence of rapid postglacial expansion by only a few haplotypes into the nonrefugial areas (Hewitt 1996, 2000, Cruzan and Templeton 2000).

It has been suggested that populations derived from separate refugia are often characterized by different haplotypes (Soltis et al. 1997, Taberlet et al. 1998). In the case of *S. praeoox*, one lineage of haplotypes (H through K) occurred in the eastern part of the Pacific Ocean side. It is possible, therefore, that the distribution range of the species has been fragmented into the eastern and the western part of the Pacific Ocean side, and that at least two refugial areas existed during the last glacial period i.e., southern part of Kyushu and/or Shikoku, and southernmost Kanto.

As the climate warmed during the postglacial period, *S. praecox* with haplotype F rapidly expanded into the northern part of the archipelago and the Sea of Japan side. In considering that some individuals with haplotype F appear in Kyushu and Shikoku, and that the haplotypes in the eastern part of the Pacific Ocean side might not have been able to expand to the Sea of Japan side due to the geological barrier of the Japan Alps (ca. 2,000～3,000m alt.) running along central Honshu, haplotype F could have expanded from the refugium in the western part of the Pacific Ocean side. In addition, it is possible that haplotype F survived at the edge of the refugial area and was the first to expand back into nonrefugial areas and occupy the available habitats, and as a consequence, other haplotype could not advance northwards (excluded by the leading edge expansion of haplotype F). The rapid leading edge expansion of a haplotype surviving at the edge of the refugial area is thought to result in reduction of haplotype diversity, and the initial colonizing population preventing the arrival of those behind them (Hewitt 1996, 2000).

The present molecular analysis resulted in an important finding for *S. sigeyosii*. This taxon was previously treated as a separate, endemic species occurring in a limestone area, Taroko region, in the northeast of Taiwan (Masamune 1938). In the more recent taxonomy of the genus *Stachyurus*, however, this species has been treated as a synonym of *S. himalaicus* (Tang 1983, Li 1993). Our results showed that *S. sigeyosii* has a unique cpDNA haplotype (N) that was nested within the *S. praecox–S. macrocarpus* clade, and not in that of *S. himalaicus* (Fig. 3). We propose two hypotheses with respect to the origin of *S. sigeyosii* and the relationship between *S. praecox* and *S. sigeyosii*. The first hypothesis is that when the Japanese archipelago was connected with the Asian continent (including Taiwan) 2 million–1.5 million years ago (Kizaki 1980), *S. praecox* or their common ancestor might be widely distributed through the Nansei Islands to Taiwan, and then, after the land bridge separated, *S. sigeyosii* might have specialize in the isolation of the limestone area. The second hypothesis is that *S. praecox* or their common ancestor might arrive in Taiwan by long distance dispersal and specialize in limestone area. It is certain that *S. praecox* is capable of long distance dispersal, given that *S. macrocarpus* exists in the oceanic Ogasawara Islands (Ohi and Murata 2000). Considering that the genus *Stachyurus* is not at present distributed in the southern part of the Nansei Islands (Okinawa Island, Iriomote Island and Ishigaki Island),

which are located between the Japanese archipelago and Taiwan, the second hypothesis seems more likely. However, the long distance dispersal of *S. praecox* could be quite rare, because haplotypes in the Japanese archipelago were not random but geographically structured.

The results of the present study support the notion that cpDNA markers, in conjunction with a thorough sampling strategy, can provide valuable insights into the intraspecific phylogeographical structure of widespread Japanese plants. Moreover, our results suggest that it is possible to interpret the process of distribution change from the last glacial period to the present in the light of phylogeographical findings. Further such research, on the phylogeographical structures of widespread plant species in the Japanese archipelago and comparison of regional patterns will provide a clearer understanding of the history of distributional change.

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大井哲雄[a], 若林三千男[b], 武 素功[c] 邑田 仁[a]：**日本列島におけるキブシ（キブシ科）の葉緑体DNAにもとづく分子系統地理**

キブシ *Stachyurus praecox* Siebold & Zucc. の分布域を通して葉緑体DNAの遺伝子間領域 *trnT*(UGU)-*trnL*(UAA)5'exon と *trnL*(UAA)5'exon-*trnF*(GAA) の塩基配列情報をもとに系統地理的構造を調べた．11種類の遺伝子変異（ハプロタイプ）が区別でき，大部分のハプロタイプが太平洋側に分布し，一方でハプロタイプFが本州北部から日本海側にかけて広域分布していた．またハプロタイプの系譜関係から，太平洋側東部地域にみられるハプロタイプが同じ系譜であった．以上をもとに，キブシの分布域は少なくとも，本州北部から日本海側にかけての地域，太平洋側東部地域，及び太平洋側西部地域，に区分できる．この葉緑体DNAの系統地理的構造は，最終氷期以降の気候変動に伴う分布域の変動を反映したものであると推測した．

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